



## ISOLATION OF ESBL PRODUCING NON-FERMENTATIVE AEROBIC BACILLI FROM STOOL SAMPLES OF HOSPITALIZED AND NON- HOSPITALIZED PATIENTS

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### ABSTRACT

**Purpose:** The aim of this study was to investigate the faecal carriage rate of TEM, SHV and CTX type ESBL producing non-fermentative aerobic bacilli (NFAB). Also these ESBLs were inspected among clinical isolates of *Pseudomonas aeruginosa* (PA) by phenotypic and genotypic methods.

**Methods:** Two hundred fresh stool samples collected from non-hospitalized and hospitalized patients were cultured on MacConkey agar supplemented with 2 mg/L cefotaxime. Non-fermentative bacteria were identified after 24 hr. incubation at 37°C by routine biochemical tests. One hundred clinical isolates of NFAB also were collected. Phenotypic tests were used to select ESBLs producing bacteria and susceptibility of isolates was determined by disc diffusion method. PCR was used to identify TEM, SHV and CTX type ESBLs producing isolates. **Results:** Six (6%), 4 (4%) and 78 (%78) bacteria from stool of inpatients, outpatients and clinical specimens were identified as cefotaxime resistance nonfermentative bacteria. All isolates of inpatients and outpatients were resistant to cefotaxime, trimethoprim sulfamethoxazole, gentamicin, kanamycin and amoxicillin-clavulanic acid while Polymyxin B and Meropenem were 100% effective. Clinical isolates were mostly sensitive to amikacin, Gentamicin, cefepim and Polymyxin B. PCR analysis showed CTX-M and SHV resistance genes in %100 (2 of 2) of inpatient isolates. None of these genes was observed in outpatient isolates. Amongst clinical isolates %35.2 (12 of 34) were identified to be positive for the blaTEM gene. **Conclusion:** Although unlike clinical isolates, stool carriage rate of CTX, SHV and TEM type ESBL producing PA among inpatients and outpatients are very low but still could be considered as a source of PA infections in hospitals.

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### Introduction

Gram-negative NFAB are responsible for many clinical infections such as septicemia, meningitis, osteomyelitis and postoperative infections. *Pseudomonas aeruginosa* (PA), *Acinetobacter baumannii* (AB), *Stenotrophomonas maltophilia* (SM) are important members of this group of bacteria (1). *Pseudomonas aeruginosa* is opportunistic pathogen bacteria and is not only responsible for nosocomial infections but also has led to outbreak of large-scale epidemics in hospitals during the last decades. Most studies have failed to find sources of environmental contamination, and thus patients were identified as potential sources of contamination (2-5). On the other hand many studies have shown sources and mechanisms of transmission of PA in patients from clinic surroundings. Moreover, it has been proved that fresh vegetables, salads and nutrition composition may be accountable for colonization of digestive tract of patients in hospital. It has also been

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demonstrated that systemic PA infections be occurred due to previous colonization of patients (6). One of the best anti-non fermentative bacteria drugs is Beta-lactam antibiotics. Non fermentative bacteria display significant resistance to antibiotics either intrinsically or attainment of resistance gen during the course of treatment (7). Metallo-Beta lactamases (MBLS) and extended-spectrum Beta lactamases (ESBLs) are resistant indicators that have high impact in clinical issues (8). In recent years, the emergence types of ESBL (CTX-M, TEM, SHV) have led to considerable resistance to expanded-spectrum cephalosporins. The production of ESBLs lead to resistance to cefotaxime, ceftazidime and aztreonam(9). Production of ESBLs in non-fermentative bacteria is one of the important ways of resistant to Beta-lactm antibiotics(10). Carriage of bacteria in healthy people is important. Admission of these carriers to hospital can lead to spread of various infections. Asymptomatic colonization of ESBL strains in fecal carriers can be as reservoir and transfer agent of these bacteria not only to hospitals but also in community. The aim of this study is to determine of ESBLs production in non-fermentative bacteria isolated from stools of admitted patients to hospital and comparison of ESBLs production with clinical isolates by phenotypic and genotypic methods in Emam Reza treatment and training center of Tabriz-2016.

## MATERIALS AND METHODS

### Bacterial strains

In between August 2016 to October 2016, 200 stool samples were gathered from hospitalized (after 48 hours of admission; n=100) and non- hospitalized patients (at beginning of admission to the hospital; n=100) from Emam Reza treatment and training center of Tabriz. Condition of receiving stool specimens from patients was lack of gastrointestinal illness (diarrhea and constipation). These were collected after receiving consent and questionnaire. One hundred nonfermentative clinical isolates were also collected regardless of patients and variety of clinical specimens. Stool samples collected were cultured in MacConkey ager containing 2mg/L cefotaxime and clinical isolates obtained by culturing different clinical specimens in MacConkey agar without cefotaxime and blood agar. Eventually all the plates were incubated at 37°C for 24 hours. Identification of the non-fermentative isolates were done by standard bacteriological test such as gram stain, triple sugar Iron agar (TSI ager), DNase, ONPG, Urea hydrolysis (11,1). Non-fermentative isolates were stored in trypticase soy broth containing %20 glycerol at-20°C.

### Determination of antimicrobial susceptibility

Antimicrobial susceptibility test of isolates were done by disk diffusion method presented by clinical and laboratory standards institute (CLSI). Using antibiotic discs such as ceftazidime (30µg), imipenem (10µg), amikacin (30µg), gentamisin (10 µg), cefotaxim, (30µg), cefoxitin (30µg), trimethoprim sulfamethoxazole (10.25µg/23.75µg), cefepime (30µg), polymyxin B (300 units), kanamycin (30µg), ciprofloxacin (5µg), tetracycline (30 µg), amoxicillin/clavulanic acid (20/10 µg), meropenem (10 µg) (Mast Group, U.K). The diameter of inhibition zone were interpreted by CLSI and manufacturer recommendations (CLSI,2015) (12).

### Determination of ESBLs producing isolates

Determination ESBL producing isolates were done using combined disc test (CDT). Bacterial suspension prepared from resistant isolates to ceftazidim and cefotaxime were cultured on Muller-Hinton agar. Disks of ceftazidim (30µg) and Ceftazidim/clavulanic (30/10 µg) and cefotaxime (CTX 30 µg), Cefotaxime/Clavulanic acid (30µg/10µg) were placed on MH plates. All the plates were incubated in 37°C for 24 hours. ESBLs were confirmed if inhibition zoon produced by the disc containing clavulanic acid were more than 5mm larger compared to the disc without inhibitor (CLSI,2015) Figure1.

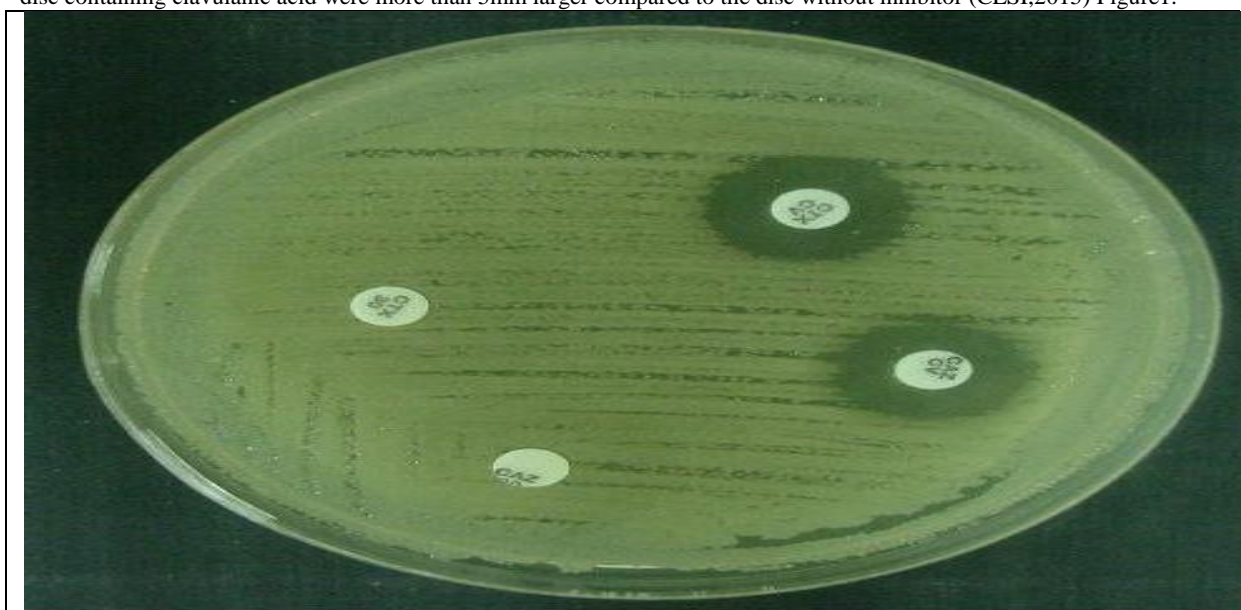


Figure1: A positive combined disc (CD) using cefotaxime (CTX 30 µg), cefotaxime/clavulanic acid (30µg/10µg) and ceftazidime (CAZ 30 µg), ceftazidime/clavulanic acid (30µg/10µg) discs. A representative of *P. aeruginosa* isolates showing

a >5 mm zone size enhancement in the CD test indicating inhibition of ESBL production.

DNA extraction was performed using sodium dodecyl sulphate-proteinase k modified with N,N,N-trimethylammonium bromide(13). PCR test was carried out by Taq master mix DNA polymerase using following primers(14):

blaCTX-M F:5'-CGCTGTTGTTAGGAAGTGTG-3'  
R:5'-GGCTGGGTGAAGTAAGTGAC-3' (569bp)  
blaSHV F:5'-CGCCTGTGTATTATCTCCCT-3'  
R:5'-CGAGTAGTCCACCAGATCCT-3' (293b)  
blaTEM F:5'-TTTCGTGTCGCCCTTATTCC-3'  
R:5'-ATCGTTGTCAGAAGTAAGTTGG-3' (403bp)

PCR condition consisted of primary denaturation (95°C, 5min), 32 cycles of denaturation (94°C, 1min), 40 seconds for annealing (57°C for TEM, 59°C for CTX<sub>M</sub>, 60°C for SHV) elongation (72°C, 40s) and final extension (72°C, 5min). All amplified products were analyzed by 1% agarose gel electrophoresis stained with safe stain and visualized by gel documentation system (UVP, USA). A 100bp molecular weight DNA ladder was used.

#### Results:

Total 6 (6%) from 100 stool samples of inpatients, 4 (4%) from 100 stool samples of outpatients and 78 (78%) from 100 clinical isolates were identified as cefotaxime resistance nonfermentative bacteria. The susceptibility data for the cefotaxime resistance non-fermentative isolates are shown in table 1.

Antibiotics	Inpatients isolates %	Outpatients isolates %	Clinical isolates %
T	66.6	100	88.1
Ctx	100	100	78
Imi	33.3	0	61.9
Pb	0	0	100
Caz	66.7	0	59.5
Ak	66.7	100	35.7
Gm	100	100	57.1
Fox	33.3	50	97.6
SXT	100	100	83.4
Cip	66.7	0	73.8
Cpm	66.7	0	59.5
Aug	100	100	95.2
Mem	0	0	69
K	100	100	92.8

T(Tetracycline, 30µg), Ctx(Cefotaxime, 30µg), Imi(Imipenem, 10µg), Pb(Polymyxin B, 300UNITS), Caz(Ceftazidime, 30µg), Ak(Amikacin, 30 µg), Gm(Gentamicin, 10µg), Fox(Cefoxitin, 30µg), SXT (Trimethoprim, sulfamethoxazole, 25µg), Cip(Ciprofloxacin, 5µg), Cpm(Cefepime, 30µg), Aug(Amoxicillin/clavulanic acid, 30µg), Mem(Meropenem, 10µg), K(Kanamycin, 30µg)

Using combined disk 2 (33.3%) from 6 fecal isolates of inpatients and 34 (43.5%) from 78 clinical isolates were identified as ESBL producing. PCR analysis showed CTX-M and SHV resistance genes in 100% (2 of 2) of inpatient isolates. None of these genes was observed in outpatient isolates. Amongst clinical isolates 35.2% (12 of 34) were identified to be positive for the blaTEM gene (Figure 2).

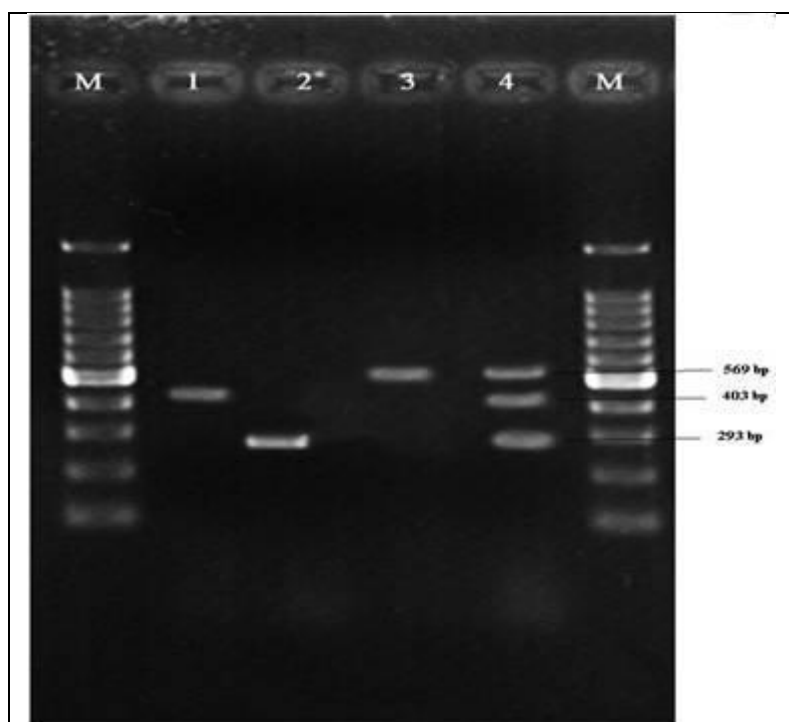


Figure2: Genotypic detection of SHV, CTX<sub>M</sub>, TEM genes in Nonfermentative bacteria. Line M is ladder .Line 1, 2, 3 are positive isolates for TEM, SHV and CTX-M respectively. Line 4 is positive controls for CTX-M, TEM and SHV.

## Discussion:

Patients attending to hospitals specially hospitalized patients are usually weak and they are very susceptible to becoming carriers of pathogenic bacteria. Bacteria can develop resistance in hospitals because of the overuse of antibiotics and as a result produce serious nosocomial infections; this is one of the important issues worldwide (15-17). In ordinary conditions without considering not only the existence of carriers of pathogenic bacteria in hospitals but also in the community, the medical workers in hospitals are usually held responsible for the outbreak of nosocomial infection (18, 19). Upon arrival carriers to a ward, they could be a risk factor of causing an outbreak of hospital and postoperative infections. Therefore, it is very important to identify carriers at the initial stage of attending to hospital. In our study six per cent of (%6) inpatients and %4 of outpatients stool samples were positive for cefotaxime resistance *Pseudomonas aeruginosa* (PA). Koichi Ohno in 2008 isolated 2.1% at hospitalization and the carrier rates of PA with a history of hospitalization were %9.1 (20). The results indicated that the rate of carriers among out patients in our research is nearly twice more than other work. But carrier rate in hospitalized patients were much lower than of reported by Koichi Ohno. The reason for this difference could be the length of time for hospitalization. Their carrier rates were significantly higher than those of the patients without any history of hospitalization. Therefore, a history of hospitalization serves as a risk factor for carrying pathogenic bacteria.

Non fermentative bacteria especially *P. aeruginosa* has now clearly emerged as a leading nosocomial pathogen, because of its ubiquitous nature, ability to survive in moist environments and acquired resistance to the vast majority of antimicrobial drugs (21, 22). However, the prevalence of ESBL-resistant *P. aeruginosa* has been increasing over recent years.

In this study, ESBLs production among nonfermentative isolated from clinical specimens was 43.59%. In a study by Tavajjohi et al. in Kashan, Iran (23), it was 9.2%. Moreover, Woodford et al.(24) in the UK and Lim et al.(25) in Malaysia reported it as 3.7% and 4.2%, respectively. However other investigators reported high prevalence of ESBLs in *P. aeruginosa* strains, for example, Zhilong Chen et al in 2015 reported variable ESBLs production in *P. aeruginosa* from 35.3% in the burn wards to 64.7% in the surgical wards showing a high different in the frequency of ESBLs production. (26)

In a survey by Yu et al in 2007 in general hospital in China 59.2% of isolates were ESBLs positive and all isolates were susceptible to imipenem (27), while our finding showed 61.9% of clinical isolates were resistant toward imipenem. The way of using antibiotics in certain circumstances, location of sampling hospital and also the studied population can explain the difference between obtained results.

The prevalence of different ESBLs genotypes varies in different countries and regions. The PER, VEB, GES TEM, SHV and CTX genotypes are prevalent in Asian countries and regions (28). They have been extensively reported in members of the family Enterobacteriaceae since the early 1980s, whereas they have been described in *P. aeruginosa* only more recently. These enzymes are either of the TEM and SHV types, which are also well known in the Enterobacteriaceae; with the PER type, mostly originating from Turkish isolates; the VEB type from Southeast Asia; and recently, the GES and IBC types, which have been reported in France, Greece and South Africa (29, 30). In Iran (Tabriz) also different type of beta-lactamase production by non- fermentative bacteria has been already reported during past decade from different specimens (31-34), but this is the first report of the presence of SHV, and CTX (33.3%) type beta-lactamases in *P. aeruginosa* isolated from carriers in our city.

The results of our investigation among inpatients and outpatients suggest that patients may indeed be a reservoir in the maintenance of non- fermentative outbreaks in various hospitals. They also can transfer resistant PA to outside community.

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